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(71) Applicant: **ABBOTT LABORATORIES** [US/US]; 100 Abbott Park Road, CHAD 0377/AP6D-2, Abbott Park, IL 60064-6050 (US).

(72) Inventors: **FESIK, Stephen, W.**; 1099 Portsmouth Circle, Gurnee, IL 60031 (US). **MEADOWS, Robert, P.**; 1745 Glenmore Drive, Green Oaks, IL 60048 (US). **BETZ, Stephen, P.**; 5830 Oberlin Drive #200, San Diego, CA 92121 (US). **LIU, Zhihong**; 5343 A David Court, Gurnee, IL 60031 (US). **OLEJNICZAK, Edward, T.**; 506 Laurie Court, Grayslake, IL 60030 (US). **SUN, Chaohong**; 390 Churchill Lane, Gurnee, IL 60031 (US).

(74) Agents: **CASUTO, Dianne** et al.; Abbott Laboratories, 100 Abbott Park Road, CHAD 0377/AP6D-2, Abbott Park, IL 60064-6050 (US).

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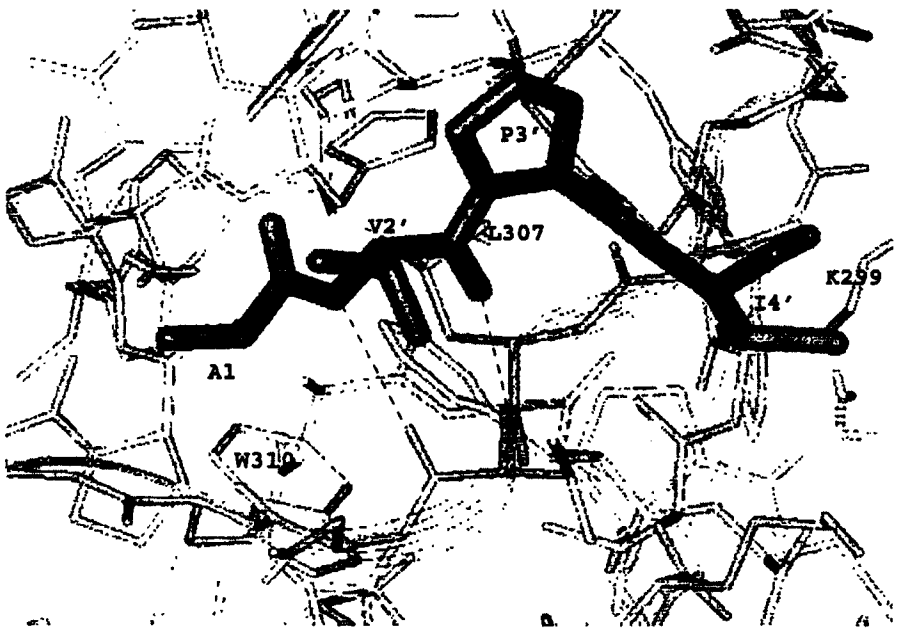
(54) Title: PEPTIDES DERIVED FROM SMAC (DIABLO) AND METHODS OF USE THEREFOR



(57) Abstract: The present invention relates to peptides derived from the wildtype human smac (DIABLO) protein which binds to a member of an IAP family member. The peptides of the present invention can be used in an assay to identify candidate substances which induce or promote apoptosis in cells.

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PEPTIDES DERIVED FROM SMAC (DIABLO) AND METHODS OF USE THEREFOR

Technical Field of The Invention

The present invention relates to peptides derived from the wildtype human smac (DIABLO) protein which bind to the inhibitor of apoptosis protein (IAP) family members, such as, but not limited to, XIAP, cIAP1, cIAP2 and survivin. The peptides of the present invention can be used in an assay to identify substances which bind to IAPs.

Background of the Invention

Apoptosis or programmed cell death (hereinafter "PCD") is a highly conserved and essential feature of development and homeostasis in higher organisms (Kelekar, et al., *Molecular and Cellular Biology*, 17(12):7040-7046 (1997)). Apoptosis is a mechanism by which the body replaces older cells with new healthy cells, or by which a cell destroys itself to prevent the transmission of genetic errors to its progeny. In some cancers, for example, it is generally accepted that an alteration in the cell growth and/or cell death is due to the accumulation of several mutations in "key" genes which regulate these processes. The normal system is unable to eliminate cells containing these mutated genes and uncontrolled cell growth results. Thus, the aberrant nature of cell growth or apoptosis observed in cancer and other diseases is the consequence of malfunctioning of the regulatory pathways which control the equilibrium between cell growth and cell death.

One class of proteins that negatively regulates programmed cell death signaling is the inhibitor of apoptosis proteins (IAPs). IAPs are highly conserved and have been found in many species (see Hay, B., et al., *Cell*, 83:1253-1262 (1995), Roy, N., et al., *Cell*, 80:167-178 (1995), Buckett, C.S., et al., *EMBO J.*, 15:2685-2694 (1996), Uren et al., *Proc. Natl. Acad. Sci. USA*, 93:4974-4978 (1996), Ambrosini, G. et al., *Nat. Med.*, 3:917-921 (1997)). Members of the IAP family include XIAP, cIAP1, cIAP2 and survivin. The members of this family are characterized by having one or more baculovirus IAP repeats called Bir domains. Bir domains consist of approximately 70 amino acids that contain the characteristic signature sequence $CX_2CX_{16}HX_6C$ (Crook, N.E., et al., *J. Virol.*, 67:2168-2174 (1993), Uren, A.G., et al., *TIBS*, 23:159-162 (1998)). Some IAPs also contain a C-terminal ring finger that contains one zinc atom chelated to three cysteines and one histidine and another zinc ligated to four cysteines (Saurin, A.G., et al., *TIBS*, 21:208-214 (1996)).

One of the major functions of the IAPs is their ability to bind to and inhibit cysteine proteases known as caspases (Hawkins, C.J., et al., *Proc. Natl. Acad. Sci. USA*, 93:13786-13790 (1996), Deveraux, Q.L., et al., *Nature*, 388:300-304 (1997)). Caspases (or cysteine-dependent aspartate specific proteases) play a key role in the execution of programmed cell death (Thornberry, N.A., et al., *Science*, 281:1312-1316 (1998)). Presently, eleven (11) human caspases have been identified, however, it is believed that others may exist (Deveraux, Q.L., *J. Clin. Immuno.*, 19(6):388-398 (1999)).

IAPs exert their anti-apoptotic effects through inhibition of caspase activity. For example, human XIAP (also called MIHA, hILP) has been shown to bind directly to caspase-3, caspase–7 and pro-caspase-9 and to inhibit apoptosis that is induced by expression of Bax or caspases (Verhagen, A., *Cell*, 102:43-53 (2000)). For human XIAP, the region responsible for inhibiting caspases–3 and –7 has been localized to a fragment containing the Bir2 domain (Takahashi, R., et al., *J. Biol. Chem.*, 273:7787-7790 (1998)), although residues outside of the Bir2 domain also have been found to be critical for inhibiting caspase–3 (Sun, C., et al., *Nature*, 401:818-822 (1999)).

XIAP also inhibits caspase–9 but not through its Bir2. Deveraux et al., (*EMBO J.*, 18:5242-5251 (1999)) have shown that the Bir3-ring finger portion of XIAP potently inhibits caspase-9, and claimed both the ring finger and the Bir3 domain of XIAP were necessary to inhibit caspase-9. However, more recently it has been shown that only the Bir3 domain is required for caspase-9 inhibition (Sun et al., *J. Biol. Chem.* (in press)) and that the earlier study had used a truncated version of the Bir-3 domain that is insufficient to cause the inhibition of caspase-9.

In addition to their interaction with caspases, IAPs also bind to proapoptotic proteins from *Drosophilia* called Reaper, HID, and GRIM. From the discovery that mutations in *Drosophila* IAP (diap 1) suppress Reaper-, HID-, and GRIM- induced apoptosis by reducing their affinity for these proteins, it is now thought that Reaper, HID, and GRIM act by preventing diap1 from binding to caspases and thereby inhibiting their activity. The major region of Reaper, HID, and GRIM responsible for binding to the IAPs and inducing

apoptosis is located at the N-terminus which is the only portion of these proteins that is similar in sequence.

Although the signaling pathways of programmed cell death are likely to be similar in *Drosophila* and mammals, until recently, no functional equivalent of Reaper, HID, and GRIM in mammals was identified. To identify mammalian proteins that bind to the IAPS, Vaux and coworkers coimmunoprecipitated a protein that bound to XIAP (MIHS) and sequenced peptides derived from this protein using electrosprayionization tandem mass spectrometry (Verhagen et al., *Cell* **102**, 43-53 (2000)). The sequence of the protein they called DIABLO (direct IAP binding protein with low pI) was identical to the sequence of a protein called smac (second mitochondria-derived activator of caspase) identified independently by Wang et al. (*Cell* **102**, 33-42 (2000)). Like Reaper, HID, and GRIM, smac (DIABLO) binds to several IAPs, inhibits IAP-mediated caspase inhibition and induces apoptosis. Thus, smac (DIABLO) could be the mammalian counterpart to the *Drosophilia* proteins Reaper, HID, and GRIM.

It is well-known that IAPs (and especially survivin) are up-regulated in cancer. Since up-regulation of IAPs results in increased binding to caspases (and a corresponding inhibition of apoptosis) there is a need for agents which competitively bind to IAPs and prevent or reduce IAP/caspase interactions. Thus, there is also a need for rapid and cost-effective screening assays to identify such agents.

Summary of the Invention

The present invention relates to an isolated and purified peptide derived from wildtype human smac (DIABLO) protein and which binds to an inhibitor of apoptosis protein (IAP) family member such as XIAP, cIAP1, cIAP2 and survivin. This peptide has the amino acid sequence:

$$\text{Ala-Xaa}_x\text{-Xaa}_y\text{-Xaa}_z\text{-(Xaa)}_n\text{-B (SEQ ID NO:1)}$$

wherein Xaa_x, Xaa_y, and Xaa_z each represent a hydrophobic amino acid independently selected from the group consisting of leucine, valine, isoleucine, phenylalanine, proline, tryptophan, tyrosine, and methionine,

n independently has a value from 0 to 20, where at least one of the Xaa_n amino acids is the same or different from that of the wildtype human smac (DIABLO) protein, and

B is absent or is a carboxy protecting group. In a preferred embodiment, at least one of the Xaa_n amino acids is different from that of the wild-type human smac (DIABLO) protein. In a more preferred embodiment, n has a value of three (3). In another more preferred embodiment, Xaa_x is valine, Xaa_y is proline, and Xaa_z is isoleucine or phenylalanine.

The present invention also relates to a peptide selected from the group consisting of:

(1) AVPIAQKSEPHSLSSEALMR (SEQ ID NO:2),

(2) AVPIAQKSE (SEQ ID NO:3),

(3) AVPIAAKSE (SEQ ID NO:4),

(4) AVPIAQASE (SEQ ID NO:5),

(5) AVPIAQKS (SEQ ID NO:6),

(5) AVPIAQK (SEQ ID NO:7),

(6) AVPIAQ (SEQ ID NO:8),

(7) AVPIA (SEQ ID NO:9),

(8) AVPIYQKSE (SEQ ID NO:10),

(9) AVPFYLPEG (SEQ ID NO:11),

(10) AVPFAQKSE (SEQ ID NO:12), and

(11) AVPFYQKSE (SEQ ID NO:13).

The present invention also relates to an assay for identifying compounds which bind to IAP family members. The first step of the assay involves providing a candidate compound to be tested. The second step involves forming a reaction mixture comprising the candidate compound and a labeled peptide derived from the wildtype smac (DIABLO) human protein having the amino acid sequence:

$$Ala\text{-}Xaa_x\text{-}Xaa_y\text{-}Xaa_z\text{-}(Xaa)_n\text{-}B \text{ (SEQ ID NO:1)}$$

wherein Xaa_x, Xaa_y, and Xaa_z each represent a hydrophobic amino acid independently selected from the group consisting of leucine, valine, isoleucine, phenylalanine, proline, tryptophan, tyrosine, and methionine,

n independently has a value from 0 to 20, where at least one of the Xaa_n amino acids is the same or different from that of the wildtype human smac (DIABLO) protein, and

B is absent or is a carboxy protecting group.

The peptide can be labeled with radioisotopic moieties, fluorescent moieties, enzymes, specific binding molecules or particles. Preferably, the peptide is labeled with a fluorescein compound, most preferably, fluorescein isothiocyanate or 5-carboxy-fluorescein. The third step involves incubating the reaction mixture under conditions sufficient to allow the candidate compound to react and bind with IAP family member. The final step involves determining whether the candidate compound has bound to the IAP family member by determining whether the labeled smac peptide has been displaced from binding to the IAP family member.

Brief Description of the Figures

Figure 1 shows the structure of the Bir3 domain of XIAP (residues 258-346) complexed to the smac peptide (Ala-Val-Pro-Ile [SEQ ID NO:14]).

Detailed Description of the Invention

1. The Present Invention

The present invention relates to peptides derived from the wildtype human smac (DIABLO) protein and which bind to a member of the IAP family of proteins. The peptides of the present invention can be used in an assay to identify substances which bind to a member of the IAP family member of proteins. Such substances can be used to promote apoptosis which could be used to kill cancer cells.

2. Sequence Listing

The present application also contains a sequence listing. For the nucleotide sequences, the base pairs are represented by the following base codes:

Symbol	Meaning
A	A; adenine
C	C; cytosine
G	G; guanine
T	T; thymine
U	U; uracil
M	A or C
R	A or G
W	A or T/U
S	C or G

Symbol	Meaning
Y	C or T/U
K	G or T/U
V	A or C or G; not T/U
H	A or C or T/U; not G
D	A or G or T/U; not C
B	C or G or T/U; not A
N	(A or C or G or T/U)

The amino acids shown in the application are in the L-form and are represented by the following amino acid-three letter abbreviations:

Abbreviation	Amino acid name
Ala	L-Alanine
Arg	L-Arginine
Asn	L-Asparagine
Asp	L-Aspartic Acid
Asx	L-Aspartic Acid or Asparagine
Cys	L-Cysteine
Glu	L-Glutamic Acid
Gln	L-Glutamine
Glx	L-Glutamine or Glutamic Acid
Gly	Glycine
His	L-Histidine
Ile	L-Isoleucine
Leu	L-Leucine
Lys	L-Lysine
Met	L-Methionine
Phe	L-Phenylalanine
Pro	L-Proline
Ser	L-Serine
Thr	L-Threonine
Trp	L-Tryptophan
Tyr	L-Tyrosine
Val	L-Valine
Xaa	L-Unknown or other

III. Peptides of the Present Invention

The present invention relates to peptides derived from the wildtype human smac (DIABLO) protein which bind to the Bir2 and Bir3 domain of XIAP. As used herein, the terms "wildtype human smac (DIABLO) protein" or "naturally occurring human smac

(DIABLO) protein" refers to the human smac protein described in Du, C., et al., *Cell*, 102:33-42 (2000) and the human DIABLO protein described in Verhagen, A., et al., *Cell*, 102:43-43 (2000). The peptides of the present invention can contain from 4 to about 25 amino acid residues, preferably from 4 to about 20 amino acids residues, and even more preferably from 4 to about 10 amino acids residues. Particularly preferred peptides are from 4 to 7 amino acid residues.

Specifically, the peptides of the present invention have the following amino acid sequence:

$$Ala\text{-}Xaa_x\text{-}Xaa_y\text{-}Xaa_z\text{-}(Xaa)_n\text{-}B \text{ (SEQ ID NO:1)}$$

wherein Xaa_x, Xaa_y, and Xaa_z each represent a hydrophobic amino acid independently selected from the group consisting of leucine, valine, isoleucine, phenylalanine, proline, tryptophan, tyrosine, and methionine,

n independently has a value from 0 to 20, where at least one of the Xaa_n amino acids is the same or different from that of the wildtype human smac (DIABLO) protein, and

B is absent or is a carboxy protecting group.

Examples of the preferred peptides of the present invention include, but are not limited to:

(1) AVPIAQKSEPHSLSSEALMR (SEQ ID NO:2),

(2) AVPIAQKSE (SEQ ID NO:3),

(3) AVPIAAKSE (SEQ ID NO:4),

(4) AVPIAQASE (SEQ ID NO:5),

(5) AVPIAQKS (SEQ ID NO:6),

(5) AVPIAQK (SEQ ID NO:7),

(6) AVPIAQ (SEQ ID NO:8),

(7) AVPIA (SEQ ID NO:9),

(8) AVPIYQKSE (SEQ ID NO:10),

(9) AVPFYLPEG (SEQ ID NO:11),

(10) AVPFAQKSE (SEQ ID NO:12), and

(11) AVPFYQKSE (SEQ ID NO:13).

The preferred peptides are optionally capped with a carboxy protecting group. As used herein, the term "carboxy protecting group" refers to a carboxylic acid protecting ester or amide group employed to block or protect the carboxylic acid functionality while the

reactions involving other functional sites of the compound are performed. Carboxy protecting groups are disclosed in Greene, "Protective Groups in Organic Synthesis" pp. 152-186 (1981), which is hereby incorporated by reference. Such carboxy protecting groups are well known to those skilled in the art, having been extensively used in the protection of carboxyl groups in the penicillin and cephalosporin fields as described in U.S. Pat. No. 3,840,556 and 3,719,667, the disclosures of which are hereby incorporated herein by reference. Representative carboxy protecting groups are C_1-C8 loweralkyl (e.g., methyl, ethyl or t-butyl and the like); arylalkyl such as phenethyl or benzyl and substituted derivatives thereof such as alkoxybenzyl or nitrobenzyl groups and the like; arylalkenyl such as phenylethenyl and the like; aryl and substituted derivatives thereofsuch as 5-indanyl and the like; dialkylaminoalkyl such as dimethylaminoethyl and the like); alkanoyloxyalkyl groups such as acetoxymethyl, butyryloxymethyl, valeryloxymethyl, isobutyryloxymethyl, isovaleryloxymethyl, 1-(propionyloxy)-1-ethyl, 1-(pivaloyloxyl)-1-ethyl, 1-methyl-1-(propionyloxy)-1-ethyl, pivaloyloxymethyl, propionyloxymethyl and the like; cycloalkanoyloxyalkyl groups such as cyclopropylcarbonyloxymethyl, cyclobutylcarbonyloxymethyl, cyclopentylcarbonyloxymethyl, cyclohexylcarbonyloxymethyl and the like; aroyloxyalkyl such as benzoyloxymethyl, benzoyloxyethyl and the like; arylalkylcarbonyloxyalkyl such as benzylcarbonyloxymethyl, 2-benzylcarbonyloxyethyl and the like; alkoxycarbonylalkyl or cycloalkyloxycarbonylalkyl such as methoxycarbonylmethyl, cyclohexyloxycarbonylmethyl, 1-methoxycarbonyl-1-ethyl and the like; alkoxycarbonyloxyalkyl or cycloalkyloxycarbonyloxyalkyl such as methoxycarbonyloxymethyl, t-butyloxycarbonyloxymethyl, 1-ethoxycarbonyloxy-1-ethyl, 1-cyclohexyloxycarbonyloxy-1-ethyl and the like; aryloxycarbonyloxyalkyl such as 2-(phenoxycarbonyloxy)ethyl, 2-(5-indanyloxycarbonyloxy)ethyl and the like; alkoxyalkyl-carbonyloxyalkyl such as 2-(1-methoxy-2-methylpropan-2-oyloxy)ethyl and like; arylalkyloxycarbonyloxyalkyl such as 2-(benzyloxycarbonyloxy)ethyl and the like; arylalkenyloxycarbonyloxyalkyl such as 2-(3-phenylpropen-2-yloxycarbonyloxy)ethyl and the like; alkoxycarbonylaminoalkyl such as t-butyloxycarbonylaminomethyl and the like; alkylaminocarbonylaminoalkyl such as methylaminocarbonylaminomethyl and the like; alkanoylaminoalkyl such as acetylaminomethyl and the like; heterocycliccarbonyloxyalkyl such as 4-methylpiperazinylcarbonyloxymethyl and the like; dialkylaminocarbonylalkyl such as dimethylaminocarbonylmethyl, diethylaminocarbonylmethyl and the like; (5-(loweralkyl)-2-oxo-1,3-dioxolen-4-yl)alkyl such as (5-t-butyl-2-oxo-1,3-dioxolen-4-yl)methyl and the like; and (5-phenyl-2-oxo-1,3-dioxolen-4-yl)alkyl such as (5-phenyl-2-oxo-1,3-dioxolen-4-yl)methyl and the like.

Representative amide carboxy protecting groups are aminocarbonyl and loweralkylaminocarbonyl groups.

Preferred carboxy-protected compounds of the invention are compounds wherein the protected carboxy group is a loweralkyl, cycloalkyl or arylalkyl ester, for example, methyl ester, ethyl ester, propyl ester, isopropyl ester, butyl ester, sec-butyl ester, isobutyl ester, amyl ester, isoamyl ester, octyl ester, cyclohexyl ester, phenylethyl ester and the like or an alkanoyloxyalkyl, cycloalkanoyloxyalkyl, aroyloxyalkyl or an arylalkylcarbonyloxyalkyl ester. Preferred amide carboxy protecting groups are loweralkylaminocarbonyl groups. For example, aspartic acid may be protected at the α-C-terminal by an acid labile group (e.g. t-butyl) and protected at the β-C-terminal by a hydrogenation labile group (e.g. benzyl) then deprotected selectively during synthesis.

As used herein, the term "loweralkylaminocarbonyl" means a -C(O)NHR^{10} group which caps the α-C-terminal of a peptide of the invention wherein R^{10} is C_1-C_4 alkyl.

As used herein, the term "aminocarbonyl" indicates a -C(O)NH_2 group which caps the α-C-terminal of a peptide of the invention.

The peptides of the present invention have been found to bind tightly to the Bir2 and Bir3 domain of XIAP.

The peptides of the present invention can be prepared using techniques known in the art. For example, a nucleotide sequence encoding a 20 amino acid residue peptide, such as for example, position 56 to 75 of the naturally occurring smac (DIABLO) protein can be synthesized using techniques known in the art. Additionally, a nucleotide sequence encoding a segment of amino acid residues from the naturally occurring smac (DIABLO) protein (and corresponding to the peptides disclosed herein) can be treated with a chemical mutagen, such as a base analog (i.e., as 5-bromouracil), a deaminating agent, or an alkylating agent (i.e., ethyl methane sulfonate (EMS)), or with a physical mutagen, such as UV or ionizing radiation or heat, using techniques known in the art.

The peptides of the present invention can also be produced by recombinant DNA techniques known in the art. For example, nucleotide sequences encoding a peptide having an amino acid sequence as described herein, can be inserted into a suitable DNA vector, such as a plasmid. More specifically, the nucleotide sequence can be inserted into a suitable DNA vector using techniques known in the art, including, but not limited to, blunt-ending or staggered-ending termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid

undesirable joining, and ligation with appropriate ligases. Techniques for such manipulations are described in Sambrook, J., et al., *Molecular Cloning: A Laboratory Manual*, 2d Ed., Cold Spring Harbor Laboratory Press, Plainview, N.Y., (1989). Once inserted the nucleotide sequence is inserted into the DNA vector, the vector is used to transform a suitable host. The recombinant peptide is produced in the host by expression. The transformed host can be either a prokaryotic or eukaryotic cell.

Alternatively, the peptides of the present invention can be directly synthesized using various solid-phase techniques (see Roberge, J.Y. et al., *Science*, 269:202-204 (1995)) and automated synthesis may be achieved, for example, using the Applied Biosystems 431 A Peptide Synthesizer.

Once the peptides of the present invention have been prepared, they may be substantially purified by preparative high performance liquid chromatography (see Crighton, T., *Proteins, Structures and Molecular Principles*, WH Freeman and Co., New York, N.Y. (1983)). The composition of any synthetic peptides of the present invention can be confirmed by amino acid analysis or sequencing (using the Edman degradation procedure). The peptides of the present invention can be used in screening assays. More specifically, when fluorescently labeled, the peptides of the present invention can be used to identify small molecules that bind to IAP family members.

As discussed earlier, the peptides of the present invention have the following amino acid sequence:

$$Ala\text{-}Xaa_x\text{-}Xaa_y\text{-}Xaa_z\text{-}(Xaa)_n\text{-}B \quad \text{(SEQ ID NO:1)}$$

wherein Xaa_x, Xaa_y, and Xaa_z each represent a hydrophobic amino acid independently selected from the group consisting of leucine, valine, isoleucine, phenylalanine, proline, tryptophan, tyrosine, and methionine,

n independently has a value from 0 to 20, where at least one of the Xaa_n amino acids is the same or different from that of the wildtype human smac (DIABLO) protein, and

B is absent or is a carboxy protecting group. Based on the NMR-derived structure of the wildtype smac peptide bound to Bir-3, amino acid residues Ala, Xaa_x, Xaa_y, and Xaa_z in the above sequence, make key contacts with the protein and thus, are necessary for tight binding to a IAP protein, such as XIAP, cIAP1, cIAP2 or survivin. However, since none of the Xaa_n residues in the above sequence make key contacts with the protein, these amino

acids may be chemically modified (e.g. fluoresceinated), without significantly affecting binding affinity. In addition, the carboxy terminus of the bound peptide, including the 4-mer peptide Ala- Xaa_x-Xaa_y-Xaa_z, does not contact the protein and therefore, it also could be modified, either directly or indirectly, via a cap ("B") such as a carboxy protecting group, without affecting binding affinity.

IV. Screening Assays Using the Peptides of the Present Invention

The present invention also relates to a variety of screening assays to identify candidate compounds that are capable of inducing or suppressing apoptosis in cells. The assay of the present invention focuses on the ability or inability of candidate compounds to disrupt the binding interaction between the smac (DIABLO) peptides of the present invention and an IAP family member protein, such as, XIAP, cIAP1, cIAP2 or survivin.

The screening assays of the present invention can be used to screen large numbers of compounds to identify those compounds which are capable of inducing or promoting apoptosis. For those compounds identified which induce apoptosis, these compounds can be used clinically for the treatment of certain cancers. Compounds which do not have activity in the screening assays can be eliminated from further consideration as candidate compounds.

The candidate compounds to be screened can encompass numerous chemical classes. However, the candidate compounds are typically organic molecules, preferably small organic compounds having a molecular weight of from about 150 to about 800 daltons. Such candidate compounds shall contain functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate compounds often contain cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate compounds can also be found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

Candidate compounds can be obtained from a wide variety of sources such as libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc., to produce structural analogs.

In competitive binding assays, the candidate compound can compete with a labeled analyte for specific binding sites on a binding agent bound to a solid surface. In such an assay, the labeled analyte can be the labeled mutant peptide derived from the wildtype smac (DIABLO) protein and the binding agent can be any IAP family protein such as XIAP, cIAP1, cIAP2 and survivin protein bound to a solid phase. Alternatively, the labeled analyte can be a labeled IAP family protein and the binding agent can be a solid phase mutant peptide derived from the wildtype smac (DIABLO) protein. The concentration of labeled analyte bound to the capture agent is inversely proportional to the ability of the candidate compound to compete in the binding assay. The amount of inhibition of labeled analyte by the candidate compound depends on the binding assay conditions and on the concentrations of binding agent, labeled analyte, and candidate compound that are used. Under specified assay conditions, a candidate compound is said to be capable of inhibiting the binding of the smac peptide to a IAP family protein in a competitive binding assay, if the amount of binding of the labeled analyte to the binding agent is decreased by ten percent (10%) or more. In a direct binding assay, a candidate compound is said to inhibit the binding of the smac (DIABLO) peptide to a IAP family protein when the signal measured is twice the background level or higher.

In a competitive binding assay, the candidate compound competes with labeled peptides of the present invention for binding to a specific binding agent (i.e. an IAP family member). As described herein, the binding agent may be bound to a solid surface to effect

separation of bound labeled protein from the unbound labeled peptides. Alternatively, the competitive binding may be conducted in a liquid phase, and any of a variety of techniques known in the art may be used to detect the release of the bound peptide or to separate the bound labeled peptides from the unbound labeled protein. Following separation, the amount of bound labeled peptides is determined. The amount of peptide present in the sample is inversely proportional to the amount of bound labeled peptide.

Alternatively, a homogenous binding assay can be performed in which a separation step is not needed. In these types of assays, binding of the labeled peptide to the protein results in a decrease or increase in the signal emitted by the label, thus allowing for quantification of the bound peptide.

An example of a competitive binding assay for detecting candidate compounds capable of inhibiting the binding of the smac (DIABLO) peptide to a IAP family protein is described in Example 1, herein. These examples describe a competitive fluorescence polarization assay where either the peptides or the synthetic compounds competed with a labeled smac peptide of the present invention for binding to the IAP family protein Bir-3.

As discussed above, the screening assays described herein employ one or more labeled molecules. The label used in the assay of the present invention can directly or indirectly provide a detectable signal. Various labels that can be used include radiolabeled compounds, fluorescent compounds, chemiluminescent compounds, bioluminescent compounds, enzymes, specific binding molecules, particles, e.g., magnetic particles, and the like. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin, etc. For the specific binding members, the complementary member is normally labeled with a molecule that provides for detection, in accordance with known procedures. Furthermore, the binding of these labels to the mutant peptides of the present invention is accomplished using standard techniques known in the art.

A variety of other reagents may also be included in the screening assay. These include reagents like salts, neutral proteins, e.g., albumin, detergents, etc., that are used to facilitate optimal protein-protein binding and/or reduce non-specific or background

interactions. Reagents that improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used. The mixture of components are added in any order that provides for the requisite binding. Incubations are performed at any suitable temperature, typically between about 0 and about 40°C. Incubation periods are selected for optimum activity. Typically, incubations from about 0.05 and 10 hours will be sufficient.

By way of example, and not of limitation, examples of the present invention shall now be given.

EXAMPLE 1: BINDING AFFINITY OF VARIANT SMAC PEPTIDES

The affinity of variant smac peptides were determined using a fluorescence polarization-based competition assay using a fluorosceinated smac peptide, smac (1-9)-FAM, AVPIAQKSEK(FAM, SEQ ID NO:21). Peptide dilutions were made against a 500 nM solution of wild-type Bir-3 (or 5 μM Bir-2) complexed with 50 nM smac (1-9)-FAM peptide buffered in PBS, pH 7.4 with 0.01% bovine gamma globulin and 0.1% sodium azide. Fluorescence polarization data were fit to a single site competition model using a non-linear least squares algorithm. The binding constant for the smac (1-9)-FAM peptide for wild-type Bir-3 was independently determined to be 0.85 μM. The results are shown below in Table 1.

TABLE I

Sequence	SEQ ID NO.	K_d for Bir-3 (μM)	K_d for Bir-2 (μM)	Ratio (Bir2/Bir3)
full-length smac		0.42 ± 0.02	2.3 ± 0.3	5.5
AVPIAQKSEPHSLSSEALMR	2	0.69 ± 0.05	6.7 ± 0.7	9.7
AVPIAQKSE	3	0.43 ± 0.06	6.0 ± 0.9	14
GVPIAQKSE		>1,000	>1,000	*
AcAVPIAQKSE	2	>1,000	>1,000	*
AAPIAQKSE	15	12 ± 2	56 ± 5	4.7
AVAIAQKSE	16	20 ± 4	4.0 ± 0.9	0.2
AVPAAQKSE	17	34 ± 7	18 ± 2	0.5
AVPIGQKSE	18	1.2 ± 0.4	10 ± 2	8.3
AVPIAAKSE	4	0.43 ± 0.08	3.5 ± 0.2	8.1
AVPIAQASE	5	0.43 ± 0.08	7.1 ± 0.6	17
AVPIAQKS-NH_2	6	0.80 ± 0.03	13 ± 3	16
AVPIAQK-NH_2	7	0.70 ± 0.09	9.4 ± 0.6	13
AVPIAQ-NH_2	8	0.8 ± 0.2	8.9 ± 0.6	11
AVPIA-NH_2	9	0.64 ± 0.07	5.5 ± 0.5	8.6
AVPFAQKSE	12	0.27 ± 0.03	14 ± 3	58
AVPIYQKSE	10	0.5 ± 0.1	2.5 ± 0.7	5.0
AVPFYQKSE	13	0.13 ± 0.03	4.9 ± 0.8	44
(n-pr)VPIAQKSE	19	>1,000	>1,000	*
(i-bu)VPIAQKSE	20	>1,000	>1,000	*

The peptides were designed to examine the sequence specificity for binding to Bir domains. As Table 1 shows, modifications of the N-terminal alanine (either by replacement with glycine or by acetylation of the alanine residue) destroyed all binding affinity for the protein. To test whether this loss of binding resulted from a steric clash with the acetyl group or the loss of the positively charged amine at the N-terminus, peptides whose N-terminal amines were substituted with a proton or methyl group (propionic or isobutyric acid vs. Ala) were tested for binding to the XIAP Bir3 domain. These peptides did not bind to the protein, suggesting that the positive charge of the Ala residue is critical for complex formation.

Mutation of the valine, proline, or isoleucine of the peptide also caused a loss of binding to the protein but to a lesser extent than the Ala substitutions. These results can be explained by a loss of hydrophobic interactions when these residues are substituted by an alanine. Amino acids C-terminal to the isoleucine do not appear to be important for binding, as peptides containing such mutations still showed tight binding to the protein. Table 1 also shows that a peptide of only 5 amino acids retained full binding affinity to the Bir3 domain of XIAP. Furthermore, peptides in which a phenylalanine residue was substituted for isoleucine displayed even tighter binding than the native residue.

The present invention is illustrated by way of the foregoing description and examples. The foregoing description is intended as a non-limiting illustration, since many variations will become apparent to those skilled in the art in view thereof. It is intended that all such variations within the scope and spirit of the appended claims be embraced thereby.

Changes can be made to the composition, operation and arrangement of the method of the present invention described herein without departing from the concept and scope of the invention as defined in the following claims.

Any references referred to herein are incorporated by reference.

WHAT IS CLAIMED IS:

1. An isolated and purified peptide derived from wildtype human smac (DIABLO) protein which binds to an inhibitor of an apoptosis protein (IAP) family member having the amino acid sequence:

$$Ala\text{-}Xaa_x\text{-}Xaa_y\text{-}Xaa_z\text{-}(Xaa)_n\text{-}B$$

wherein Xaa_x, Xaa_y, and Xaa_z each represent a hydrophobic amino acid independently selected from the group consisting of leucine, valine, isoleucine, phenylalanine, proline, tryptophan, tyrosine, and methionine,

n independently has a value from 0 to 20, where at least one of the Xaa_n amino acids is the same or different from that of the wildtype human smac (DIABLO) protein, and

B is absent or is a carboxy protecting group.

2. The peptide of claim 1 wherein the IAP family member is XIAP, cIAP1, cIAP2 or survivin.

3. The peptide of claim 1 wherein Xaa_x is valine, Xaa_y is proline, and Xaa_z is isoleucine or phenylalanine.

4. The peptide of claim 1 or claim 3 wherein n has a value of three (3).

5. A peptide selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12 and SEQ ID NO:13.

6. An assay for identifying substances which bind to an IAP family member, the assay comprising the steps of:

(a) providing a candidate substance to be tested;

(b) forming a reaction mixture by contacting the candidate substance with an IAP family member and a labeled peptide derived from the wildtype human smac (DIABLO) protein having the amino acid sequence:

$$Ala\text{-}Xaa_x\text{-}Xaa_y\text{-}Xaa_z\text{-}(Xaa)_n\text{-}B$$

wherein Xaa_x, Xaa_y, and Xaa_z each represent a hydrophobic amino acid independently selected from the group consisting of leucine, valine, isoleucine, phenylalanine, proline, tryptophan, tyrosine, and methionine,

n independently has a value from 0 to 20, where at least one of the Xaa_n amino acids is the same or different from that of the wildtype human smac (DIABLO) protein, and

B is absent or is a carboxy protecting group,

(c) incubating the reaction mixture under conditions sufficient to allow the candidate substance to react and bind with the IAP family member; and

(d) determining whether the candidate substance has bound to the IAP family member by determining whether the labeled smac (DIABLO) peptide has been displaced from binding to the IAP family member.

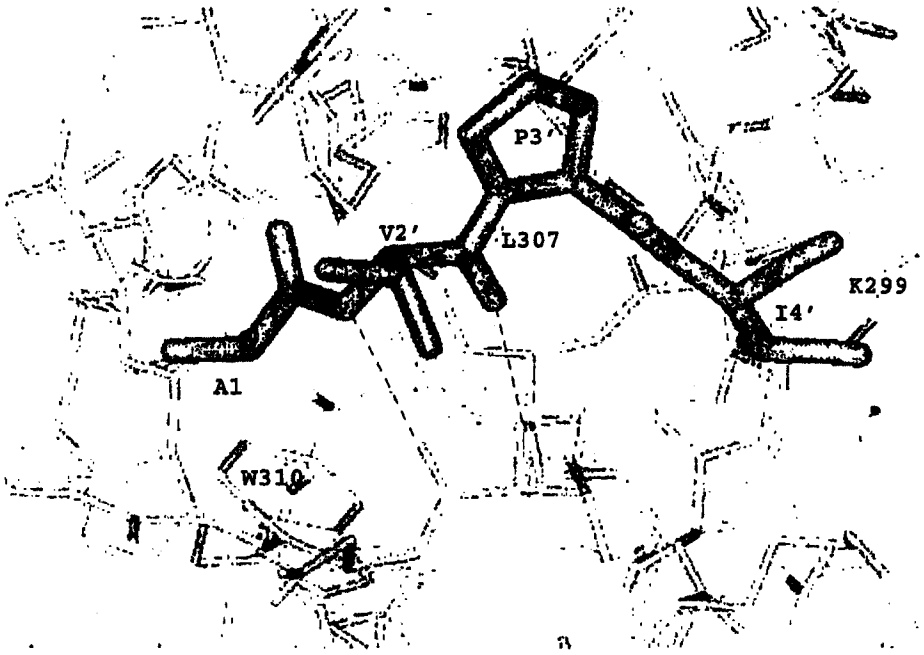
7. The assay of claim 6 wherein the IAP family protein is XIAP, cIAP1, cIAP2 or survivin.

8. The assay of claim 6 wherein the peptide is selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12 and SEQ ID NO:13.

9. The assay of claim 6 wherein Xaa_x is valine, Xaa_y is proline, and Xaa_z is isoleucine or phenylalanine.

10. The assay of claim X or claim Y wherein n has a value of three (3).

Figure 1



SEQUENCE LISTING

<110> Fesik, Stephen W.
Betz, Stephen F.
Meadows, Robert P.
Liu, Zhihong
Olejniczak, Edward T.
Sun, Chaohong

<120> PEPTIDES FROM SMAC (DIABLO) AND METHODS OF USE THEREFOR

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